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Award Number: W81XWH-06-1-0085

TITLE: Monoclonal Antibodies to Prevent Use of Mycotoxins as Biological Weapons

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REPORT DATE: July 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-07-2007	Final	5 Dec 2005 – 30 Jun 2007

4. TITLE AND SUBTITLE

Monoclonal Antibodies to Prevent Use of Mycotoxins as Biological Weapons

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0085

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Albert Einstein College of Medicine
Bronx, NY 10461

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Aflatoxin exposure causes a broad range of adverse effects, including acute hepatic failure, hepatic carcinoma and immunosuppression. The ability to weaponize aflatoxins has already been demonstrated, raising concern that these potent agents might be used for biowarfare or agroterrorism. Passive antibody therapy is used for the treatment of toxin exposures and its potential for use in the event of bioterrorism has been highlighted. Monoclonal antibodies have been made to aflatoxins, but, currently, none have been demonstrated to neutralize the adverse effects of exposure. The research goal is to generate monoclonal antibodies to aflatoxins for study of their potential to prevent disease. We generated a novel aflatoxin conjugate using keyhole limpet hemocyanin as a carrier and used this conjugate to immunize rats. We found that vaccination by either the intraperitoneal or subcutaneous route induced very high aflatoxin B1-binding antibody titers that were completely competed by free aflatoxin BB1 in solution. We will make and characterize monoclonal antibodies to aflatoxins B1 and G1 and test them for their ability to inhibit cytotoxicity to rat hepatocytes and macrophages in vitro. Protective MAbs would subsequently be further developed for passive therapy in humans in the event that aflatoxins are used as agents of biowarfare.

15. SUBJECT TERMS
Aflatoxin, monoclonal antibodies, biowarfare

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	7	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION:

Aflatoxins are small molecule secondary metabolites of the fungi *Aspergillus flavus* and *Aspergillus parasiticus* that are potent hepatic carcinogens and have a variety of acute and chronic toxic effects, including acute hepatic necrosis. Concern regarding their use as agents for biowarfare or agroterrorism arose following demonstration of their ability to be weaponized (6). Passive administration of monoclonal antibodies (MAbs) is used for toxins and their potential for use in the event of bioterrorism has been highlighted (1). Immunization with an aflatoxin B_1 (AF-B_1)-BSA conjugate previously had shown protective efficacy against subsequent exposure in rabbits, with reduced mortality and hepatotoxicity in survivors (7). However, given the potential toxicity of vaccine administration, this strategy is unlikely to be of use in humans. Monoclonal antibodies (MAbs) that are able to neutralize aflatoxin toxicity have not been reported. Therefore, we proposed to make rat MAbs to AF-B_1 and AF-G_1 and to conduct preliminary efficacy testing in vitro.

BODY:

Production of novel aflatoxin conjugate. Because MAbs made to the previously reported aflatoxin-BSA conjugate had not been reported to be protective, we made a novel conjugate of aflatoxin B_1 to KLH. A carboxymethyloxime group was introduced into aflatoxin B_1, leaving the terminal furan ring that forms the highly reactive AFB_1-8,9-epoxide unmodified (2). AFB_1-1-(*O*-carboxymethyl) oxime was purified by high performance liquid chromatography using an Adsorbosil-5 column. The identity of the product was confirmed by mass spectrometry (**Figure 1**). The AFB_1-1-(*O*-carboxymethyl) oxime then was conjugated to mariculture Keyhole Limpet Hemocyanin (KLH) using the Pierce Imject Immunogen EDC kit. The molar ratio, as determined by Bradford assay and AF-B_1 competition ELISA using purified AF-B_1 ranged from 10 to 50 (AF-B_1:KLH).

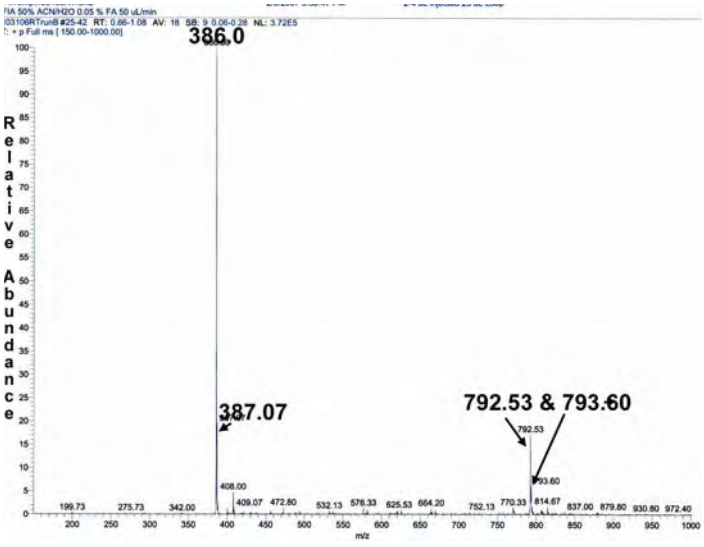

Figure 1. Mass map from mass spectrometry of AF-B_1-(*O*-carboxymethyl) oxime. The peaks at 386 and 387.07 are consistent with AF-B1-(*O*-carboxymethyl) oxime. The smaller peaks at 792.53 and 793.60 are consistent with AF-B1-(*O*-carboxymethyl) oxime dimers. Aflatoxin standard produced peaks at 313.20 and 314.20 (not shown).

AF-B_1-KLH conjugate vaccine is potently immunogenic. Rats were selected for use because of their higher susceptibility to the harmful effects of aflatoxins in comparison to mice (3). Female Fischer rats were bled orbitally and then immunized with 50 μg of conjugate emulsified in complete Freund's adjuvant, administered subcutaneously (s.c.) or intraperitoneally (i.p., three rats each). Control rats received buffer or pure KLH s.c. or i.p. Fourteen days later, rats again were bled and then boosted with a second dose of conjugate without KLH. AF-B_1-binding antibody titers were determined by ELISA using commercially available AF-B_1–bovine

serum albumen conjugate to coat the microtiter plate, since AF-B_1 alone does not bind. The titer was defined as the dilution at which the A_{405} was greater than two times that of background. As shown in **Figure 2**, five of the six rats that received conjugate vaccine mounted very high AF-B_1–binding IgG titers. Maximum serum IgM titers titers were obtained on day 14, when the geometric mean inverse titer was 600 (range: 400-1600) and had declined by day 28. No difference was seen on the basis of route of vaccine administration. No AF-B_1 binding was detected in any of the control rats (not shown). KLH-binding antibody titers in rats that received the conjugate vaccine were substantially lower.

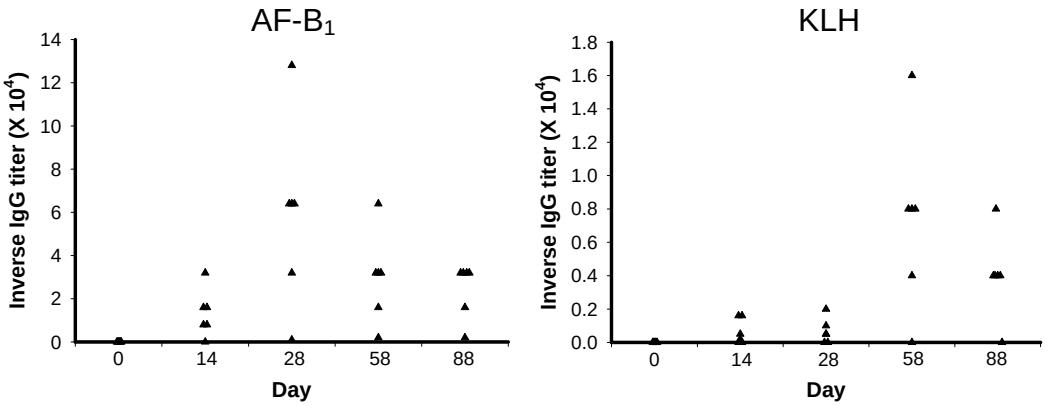

Figure 2. Antibody titers following AF-B_1-KLH conjugate vaccine to AF-B_1 (left) or KLH (right). Rats were vaccinated on day 0 and boosted on day 14. Each triangle represents one rat, n = 6. The limit of detection was a titer of 1:50. Note scale difference between the two panels.

We wanted to ensure that AF-B_1 binding was specific for the AF-B_1 portion and not the carboxymethyl oxime portion of the molecule, as the same method was used to construct the BSA conjugate used in our ELISA. Therefore, competition ELISAs were done using sera obtained 28 days after the initial vaccination. As seen in **Figure 3**, antibody binding to the AF-B_1-BSA conjugate was completely inhibited by the presence of free AF-B_1 in solution, indicating that AF-B_1 was bound. No binding was seen when pre-immune sera were used in this assay (not shown).

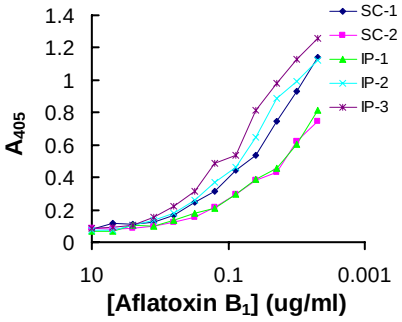

Figure 3. Competition ELISA to demonstrate specificity of antibody binding for AF-B_1. Microtiter plates were coated with AF-B_1-BSA conjugates, then blocked and then free AF-B1 was added in solution and diluted across the plate. IgG binding of the serum from the five rats that mounted antibody responses to the conjugate vaccine is shown.

As seen in Figure 2, antibody titers to AF-B_1 remained high 88 days after vaccination. Though we are very excited by the immunogenicity of our vaccine, standard hybridoma technology necessitates that antibody titers come down prior to boosting and fusion, as plasma cells are terminally differentiated and do not fuse because they are not cycling. Because of the toxicity of AF-B_1, we cannot give massive doses to exhaust serum antibody titers prior to boosting to stimulate memory B cell

responses. Thus, we are continuing to follow antibody titers on a monthly basis. However, the persistently high titers may result from ongoing antigenic stimulation due to co-administration of complete Freund's adjuvant with the initial vaccination. Thus, we are also vaccinating a few additional rats with a single dose of conjugate alone to see if we can get a sufficiently high titer and rapid decline to allow us to move forward. An additional advantage would be that, even with CFA, rats did not begin to develop detectable titers to KLH until after 28 days. Thus, though we plan to screen hybridomas for both AF-B1 and KLH binding, we may obtain a higher percentage that has our desired specificity. An additional option would be to use a fusion partner transfected to overexpress *bcl-2* that, therefore, is resistant to apoptosis, resulting in its ability to fuse to a broader range of B cells (4). This cell line is available to us at Albert Einstein College of Medicine. However, cell lines made with this partner are commonly less stable. Therefore, we will use this option only if the other methods are unsuccessful. A final possibility would be to stimulate splenocytes ex vivo prior to fusion. However, because of biosafety concerns related to aflatoxin handling, we are avoiding this option, as well. Nonetheless, we are confident that one of these options will be successful in the near future.

KEY RESEARCH ACCOMPLISHMENTS:

- Synthesis of a novel AF-B_1-KLH conjugate vaccine
- Demonstration of potent immunogenicity of the conjugate vaccine

REPORTABLE OUTCOMES:

None. Development of cell lines and publication are anticipated.

CONCLUSION:

We are committed to completing the studies in the statement of work for this contract. Therefore, I requested an additional no-cost extension on June 12, 2007 and am awaiting notification regarding the status of this request. In addition to planning the AF-B_1 fusion in the near future, we have begun constructing the AF-G_1 conjugate. We will make MAbs to the two aflatoxins. Aflatoxin-binding MAbs will be characterized on the basis of isotype, affinity for each aflatoxin type, variable region sequencing and competition for binding with each other. We will test MAbs for their ability to prevent toxicity to hepatocytes and alveolar macrophages. Cytotoxicity will be assessed by lactate dehydrogenase release assay and by microscopy (5). Unscheduled DNA synthesis will be used to measure DNA damage (8). Protective MAbs would subsequently be further developed for passive therapy in humans. We believe that these studies have the potential to produce biologicals that would be able to provide specific prophylaxis or therapeutic benefit of prolonged duration should aflatoxin be used as an agent of biowarfare.

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APPENDICES:

None.

SUPPORTING DATA:

See text.